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13. ABSTRACT ***(Maximum 200 Words)***

The purpose of this project was to immunopurify genomic DNA fragments bound by BRCA1 protein in vivo. Likely isolated DNA fragments were to be tested in a reporter system to determine if they confer BRCA1-dependent expression, and analyzed in gel shift assays and DNase I footprinting experiments to determine if they were bound by BRCA1 in vitro. We have made significant gains in achieving the objectives of this project during the past (second) year of funding. Our accomplishments include: 1) the construction of a library of immunopurified DNA fragments cloned into a luciferase reporter vector; 2) identification of at least three independent clones that appear to switch on reporter gene expression when BRCA1 is over-expressed; 3) sequence analysis of the aforementioned clones which has revealed that one of them (clone 3-9) contains an unusual repeat structure containing binding sites for multiple known transcription factors; 4) evidence, based upon Southern hybridization, that DNA fragments conferring BRCA1 regulation contain related sequences. Further characterization of identified clones and complete screening of our library is expected to yield important new information on the function of the crucial BRCA1 protein.

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Susan Austin 10/12/99

PI - Signature Date

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INTRODUCTION

The subject of this report is the protein product of the breast-ovarian cancer susceptibility gene, BRCA1. Inherited mutations in BRCA1, that disrupt the synthesis of the wild-type BRCA1 protein, are responsible for about half of hereditary breast cancer families and most families that contain multiple cases of breast and ovarian cancer (1). Current estimates of cancer risk associated with germline mutations of BRCA1 are 56% for breast cancer and 16% for ovarian cancer by age 70 years (2). Our research is concerned with investigating the function of the BRCA1 protein and specifically, to determine if BRCA1 is a DNA binder and is able to regulate the expression of target genes. Our plan was to develop a regulated expression system, to conditionally express an epitope-tagged BRCA1 protein, so that we could immunopurify BRCA1-DNA complexes from solubilized chromatin fragments using an antibody that recognizes the epitope tag. However, we were stymied in this approach during our first year since we were not successful in our attempts to make the relevant cell lines. However, we were successful in making our own antibody which specifically detects BRCA1 protein allowing us to continue with our experiments. This report describes our accomplishments during the past (second) year of funding which have yielded significant gains in our objective to isolate genomic DNA fragments bound by BRCA1 protein in vivo.

BODY

Immunoprecipitation of BRCA1-DNA complexes.

Complexes of BRCA1 protein bound to genomic DNA fragments were isolated from the breast cancer line MCF7/6 using an immunoprecipitation procedure. Briefly, cells were grown to near confluence in 100 mm dishes and washed twice with phosphate buffered saline (PBS). A solution of ice cold 10 mM Tris pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% Nonidet P-40, containing a cocktail of protease inhibitors, was added to the cells and the cells scraped from the dish using a rubber cell scraper. Up to 14 dishes of cells were processed at one time. Cells were transferred to a Dounce homogenizer where they were lysed, with care to keep the lysate cold at all times. The lysate was then layer over a cushion of 30% sucrose and centrifuged at 2000xg for 5 minutes. Cell debris collected at the interface of the sucrose cushion while nuclei collected at the bottom of the tube. Isolated nuclei were washed with lysis solution and centrifuged through a second sucrose cushion to remove any contaminating debris. We found that the quality of immunoprecipitated DNA (see later) depended greatly upon keeping the nuclei preparation cold at all times and including protease inhibitors in all of the solutions.

Isolated nuclei from 14 dishes were resuspended in 1.0 ml of React buffer #2 (Life Technologies) and incubated with 600 Units of Hae III restriction enzyme for 30 minutes at 37°C. The digested nuclei were harvested by centrifugation at 2000xg for 2 minutes and resuspended in 0.7 ml of 12 mM Tris-Acetate (pH 7.5) and 1 mM EDTA, and incubated on ice for 30 minutes. The nuclei were harvested by centrifugation at 1800xg for 5 minutes and the eluted chromatin fragments transferred to a fresh tube. Sodium chloride and bovine serum albumin were added to final concentrations of 100 mM and 1 mg/ml, respectively.

Prior to immunoprecipitation of specific BRCA1-DNA complexes, the chromatin fragments were pre-cleared by incubation with 5 μg of pre-immune IgG and 70 μl of protein A-agarose beads for 1 hour at 4°C. The beads were removed by brief centrifugation and 5 μg of anti-BRCA1 antibody added (# 5600; see previous report) and incubated for 2 hours at 4°C. Next, 70 μl of protein A-agarose beads were

added and the incubation continued for a further 1 hour. The beads, to which BRCA1-DNA complexes were bound, were harvested by brief centrifugation and washed twice with ice-cold Tris-acetate buffer (pH 7.5). Finally, the beads were resuspended in 200 µl of 10 mM Tris (pH 8.0) and 1 mM EDTA and the isolated DNA fragments purified by phenol-chloroform extraction and ethanol precipitation.

From a typical preparation we isolated approximately 100 ng of genomic DNA fragments that had a size range of 300 to 2000 base-pairs (bp). Importantly, the DNA fragments formed a ladder when analyzed by gel electrophoresis with a step-wise increase in size of about 150 bp, consistent with the periodicity of DNA packaged into nucleosomes.

Construction of a library of BRCA1-bound DNA fragments.

In the original grant application I stated that isolated DNA fragments would be subjected to DNA sequencing depending upon the number and size of fragments. This was not a good idea and a criticism of the grant on peer review was that most of the isolated DNA fragments would not, in fact, be bound by BRCA1 protein in vivo and that a screening step would be needed, to select for relevant fragments, prior to DNA sequencing. We have taken this advice and, after careful consideration, decided to screen individual fragments for their ability to activate a reporter construct when co-transfected with BRCA1. Other laboratories, that have used the same immunoprecipitation technique, have used protein-binding to screen for relevant DNA fragments, but we were not able to do this since firstly, we do not have purified full-length BRCA1 protein at this time and secondly, there is no guarantee that pure BRCA1 will bind DNA (additional proteins are likely to be required). Instead, we elected to clone isolated DNA fragments into a luciferase reporter plasmid; construct a library of isolated DNA fragments; and screen pools of clones for their ability to activate luciferase gene expression in the presence of BRCA1.

We chose the pGL3-Enhancer vector from Promega in which to clone isolated DNA fragments. This plasmid carries the luciferase reporter gene, which allows for extremely sensitive assays of gene expression, and an SV40 enhancer element, downstream of the reporter, which aids the identification of functional promoter elements giving rise to higher levels of luciferase expression. Our hypothesis was that in vivo DNA fragments bound by BRCA1 were likely to be promoter elements from target genes and therefore the screening of DNA fragments for BRCA1-regulated transcription seemed appropriate and fits well with the aims of the project (see specific aim #3).

Isolated genomic DNA fragments were cloned into the Sma I site of the pGL3-Enhancer vector. DNA preparations from 20 independent clones revealed that 19 of them were recombinant and contained inserts from 300-2000 bp. Nearly 3,000 colonies were picked and stored in 96 well plates, forming our library to screen for BRCA1-regulated clones. Additional ligation reaction was frozen should we need, in the future, to screen further clones.

Screening of isolated DNA fragments for BRCA1-dependent transcription.

Library clones containing putative BRCA1 targets were screened for BRCA1-dependent transcription in pools of 16 clones, aiding the rapid analysis of DNA fragments. In total, we have isolated plasmid DNA from 180 pools, representing 2,880 immunopurified clones. Thirty of these pools have now been analyzed for BRCA1-dependent transcription by co-transfecting MCF7/6 cells either with pool DNA plus BRCA1 expression plasmid or with pool DNA plus empty expression plasmid. Transfections were performed in duplicate and the mean obtained, +/- BRCA1 over-expression, for each

pool. We set an arbitrary figure of 2.0 as the level of luciferase gene induction beyond which pools would be analyzed further. This number corresponds to a biologically significant level of reporter gene induction and allows for some variability in assay measurements related, say, to transfection efficiency and experimenter error. Pools with a level of luciferase induction of 2.0 or greater (in the presence of BRCA1 over-expression) were assayed a second time to determine if the results were reproducible. Using these criteria, of 30 pools screened to date, six appear to contain clones that switch on reporter gene expression when BRCA1 is over-expressed (Table 1).

Controls were, and continue to be, an important part of this screening process. Clearly, the objective was to compare reporter gene expression in the presence of different levels of BRCA1 protein so that we could evaluate which clones contained BRCA1-responsive fragments. Since MCF7/6 cells contain endogenous BRCA1 protein we expected to see reporter gene expression even when BRCA1 was not over-expressed, but depending upon the strength of the promoter fragment cloned, the amount of luciferase activity was expected to vary between different fragments. We routinely measured BRCA1 protein levels on western blots to ensure that BRCA1 was being over-expressed in the relevant transfected cells. We also performed transfections with empty pGL3-Enhancer vector and found little difference in luciferase activity +/- BRCA1 over-expression. The finding that most pools in fact did not have elevated reporter gene activity in the presence of BRCA1 over-expression suggested that pools which did show up-regulation in response to BRCA1 did so because of a cloned insert. Finally, we were also receptive to the idea that some DNA fragments may repress reporter expression in the presence of high levels of BRCA1 but so far we have only seen up-regulation.

To identify single clones that contained BRCA1-responsive DNA fragments we prepared DNA from individual clones from two of the positive pools. In pool 3, one clone (number 9) up-regulated luciferase expression over 3-fold when BRCA1 was over-expressed (Table 2). The level of expression of this clone, named 3-9, was much greater than that of any of the other clones in this pool, indicating that it did indeed contain a promoter-like element and, upon repeated testing, up-regulated luciferase expression in the presence of high levels of BRCA1. Note that several other clones in this pool appeared to show BRCA1 regulation but their level of reporter gene expression was so low that the observed fold induction was probably unrelated to BRCA1, but due to the intrinsic variability associated with the assay system. Individual clones from pool 4 were similarly tested and one clone, number 7, had high luciferase activity with and without BRCA1 over-expression, suggesting that it contained a promoter element unrelated to BRCA1 function. A second clone, number 10, increased luciferase expression over 3-fold suggesting that it also conferred BRCA1-dependent regulation.

Sequence analysis of positive clones.

Characterization of clone 3-9. Excision of the DNA insert from clone 3-9 revealed that it was approximately 950 bp long. Restriction mapping revealed the presence of a Pvu II site almost exactly midway along the length of the insert and so to narrow the promoter-like activity more precisely we used the Pvu II site to make deletion constructs (Figure 1). The 5' most half of the insert (with respect to the reporter) had little luciferase activity, but the 3' portion strongly promoted reporter gene expression and was responsive to BRCA1.

DNA sequencing of the entire insert from clone 3-9 revealed that it was chimeric, containing two internal Hae III restriction sites. A single Hae III fragment spanned the Pvu II site extending to the cloning site adjacent to the luciferase reporter gene. DNA sequencing revealed that this fragment was

almost identical to a portion of a human collagen gene except that a 24 bp fragment was repeated 10 times. The finding that we had identified a portion of a human collagen gene was, at first, disappointing since biologically it was not a plausible candidate for BRCA1 binding. The region of the collagen gene identified was close to the middle of the gene and most of it, with the exception of a small exon, was intronic. Database searches have not revealed the presence of any other expressed sequences present within, or close to the cloned fragment, such as within any of the collagen introns or on the non-coding strand. The repeated sequence is present in Genbank only once and there is no indication that this gene contains a polymorphism, such as a variable number of tandem repeats (VNTR), in the vicinity. Careful examination of the sequence did not reveal any Hae III sites, indicative of a cloning artifact, and the bacterial host used for constructing our library was recombination negative. Currently, we are examining the MCF7/6 cell line from which this DNA fragment was isolated to ensure that the amplification is present in genomic DNA and are analyzing additional human DNAs to determine whether it is polymorphic. Certainly, at this time, we have not ruled out the possibility that the observed amplification is a somatic mutation since the immunopurified fragments were derived from a cancer cell line.

We also have analyzed the sequence of the 3-9 clone for transcription factor binding sites using the MatInspector program available on-line. The repeat unit in the 3-9 clone contains binding sites for Sp1 and c-Ets-1 (p54) itself, but two units joined head to tail form 7 additional binding sites for the same and other transcription factors (Figure 2). Ten such repeats, as found in clone 3-9, likely represents a "hot-spot" for transcription factor binding and almost certainly is responsible for the strong promoter-like activity that we observed. The critical question right now, as we see it, is how BRCA1 up-regulates reporter gene expression from clone 3-9, and we plan gel shift assays and DNase I footprinting experiments to test whether BRCA1 binds the 3-9 clone and, if so, to identify the binding site(s). I realize that gel shift assays are ahead of reporter assays in my Statement of Work but we chose to do reporter assays to screen for relevant immunopurified clones. Clearly, our recent results raise some very intriguing questions which we are being to address as this report is being written.

Characterization of clone 4-10. The insert from clone 4-10 was 1058 bp long and, upon BLAST sequence analysis, revealed an exact match to a genomic DNA fragment from chromosome 19. The fragment is not chimeric and does not contain any Alu repeats. Like the 3-9 clone, we are performing gel shift assays to determine if BRCA1 protein binds to the insert DNA present in clone 4-10. A search of the 4-10 sequence reveals many transcription factor binding sites, some of which are common to clone 3-9 (Table 3). It is difficult to determine which, if any, may be functionally significant since BRCA1 may have its own signature binding sequence or be a co-activator with a separate protein. However, it is probably correct that Sp1 sites are not relevant here since 3-9 has 29 Sp1 sites while 4-10 has none. In contrast, both of the clones contain transcription factor binding sequences for AP4, c-Ets-1(p54) and deltaEF1, to name but a few. Gel shift assays and DNase I footprinting experiments will help resolve which of the identified binding sites or a novel BRCA1 binding sequence is relevant to explain the observed reporter activities.

<u>Hybridization of the 450 bp promoter fragment from clone 3-9 to DNA from pools 1-40</u>.

We wanted to determine if the insert from clone 3-9 had been recovered multiple times in our library of immunopurified fragments. To test this possibility, we purified the 450 bp fragment containing the promoter activity and used it as a hybridization probe on plasmid DNA prepared from the first 40

pools. Southern blotting showed strong hybridization only to pool #3 (which contains the 3-9 clone), indicating that there were no other clones with sequence identity to 3-9 within the first 40 pools, but weak hybridization to clones from pools 4, 26, 29, 39 and 40 (Figure 3). Importantly, we noted that pools 4, 26 and 29 were positive in reporter assays for BRCA1-dependent transcriptional regulation, suggesting a correlation between hybridization and reporter activity and consistent with this, the hybridizing fragment in pool 4 was shown to be derived from clone 4-10. This result suggested that clones 3-9 and 4-10 share sequence homology although BLAST database searches had previously shown that they were derived from different regions of the genome.

To identify the hybridizing clone in pool 26 we analyzed each of the clones by Southern blotting as we had done for the first 40 pools. A single positive clone, 26-13, was detected (Figure 4) which we tested in reporter assays, +/- BRCA1 over-expression. Without BRCA1 we recorded a measurement of 24, but when BRCA1 was over-expressed activity increased to 60, suggesting that we had identified a third fragment conferring BRCA1-dependent reporter expression. Restriction digests revealed that the insert in 26-13 was at least 2000 bp long and, to date, we have sequenced about 700 bp from either end. BLAST sequence analysis revealed no exact matches to sequences in Genbank but one end of 26-13 did contain repetitive/Alu DNA.

Since the promoter-like fragment from clone 3-9 hybridized albeit weakly to 4-10 and 26-13 we decided to compare the sequence of each with each other to determine if they contained significant homology. We used the BLAST server to perform this function and adjusted the penalties for mismatches and gaps to become less stringent. However, we failed to detect any homology between 3-9, 4-10 or 26-13, except for a 35 bp repeat region found in 4-10 and one end of 26-13. Note that we have not yet completed the sequencing of 26-13 so we were only able to include the ends of this clone in the comparison. One explanation for our findings is that since clone 3-9 contains 10 copies of a repeat, forming multiple copies of several transcription factor binding sites, the hybridization that we observed is, in fact, due to these repeats, only in clones 4-10 and 26-13 the repeat sequence is not a perfect match and copies of this repeat may be scattered throughout the insert.

The preliminary data that we have generated suggests that our library of immunopurified DNA fragments, immunoprecipitated using our own anti-BRCA1 antibody, does indeed contain clones that confer BRCA1-dependent reporter gene expression. Sequence analysis of several clones reveals that one of them contains an unusual repeat structure, associated with a known gene, which we are investigating further, while two others clearly contain human DNA but are not known to be associated with actively transcribed genes. Other positive clones are present in our library but have not yet been analyzed further.

KEY RESEARCH ACCOMPLISHMENTS

- We have created a library of genomic DNA fragments immunopurified using an antibody raised to BRCA1.
- Reporter assays have begun to identify individual clones that contain promoter-like activity and which up-regulate luciferase gene expression when BRCA1 protein is over-expressed.
- Sequencing of one of the identified clones, 3-9, has revealed ten near perfect copies of a 24 bp segment containing multiple transcription factor binding sites. We are currently in the process of

determining whether the identified repeats are due to a VNTR polymorphism or represent the product of a likely somatic mutation.

- Sequence analysis of additional clones reveal binding sites for AP2, AP4, c-Ets-1, DELTA EF1 GC, GKLF, Ik2 and Sp1. Gel shift assays and footprinting experiments will be used to determine which, if any of these sites, are bound by BRCA1 protein.
- Southern blotting indicates that clones containing BRCA1-regulated promoter-like activity share DNA sequence homology, though searches of the sequences have failed to pinpoint what this homology might be.

REPORTABLE OUTCOMES

Manuscripts: None yet. If the gel shift assays that we are performing demonstrate BRCA1-DNA binding we will write a manuscript.

Patents: None.

Degrees obtained: None.

Development of cell lines etc.: We have constructed an MCF7/6-Tet-Off cell line allowing conditional expression from a regulatable promoter. We are using this cell line for our reporter assays. We have also constructed a library of immunopurified BRCA1 DNA fragments.

Informatics: None.

Funding applied for:

American Cancer Society Project Grant (October, 1999).
Department of Defense ovarian cancer Idea Grant (October, 1999).

Employment or research opportunities applied for and/or received: Jason Schmittschmitt, who was a research technician supported by the grant during the first year of funding, got into graduate school at the University of Texas (at College Station) based, in part, upon research experience he gained while working on the described project.

CONCLUSIONS

We have begun to isolate likely genomic DNA fragments that encode promoter-like activity and which show BRCA1-dependent regulation. Screening of a library of immunopurified fragments, isolated using an antibody which we developed to BRCA1, has identified independent clones which contain binding sites for several transcription factors. Gel shift assays and DNase I footprinting experiments being performing at the writing of this report will reveal whether BRCA1 binds directly or indirectly with DNA and if so, allow us to identify a specific binding site. The importance of this work with respect to breast cancer is that BRCA1 is a hereditary breast cancer gene and understanding the function of BRCA1, especially its tumor suppressor activity, will deepen our knowledge of how breast cancer develops. Since little is still known about the etiology of sporadic breast cancer, the genes that cause hereditary cancer hold important clues to understanding breast cancer development in general especially

since BRCA1 has been implicated in sporadic cancers as well as the less common hereditary cancers. And yet despite intense research, concentrated principally on what other proteins BRCA1 interacts with, still little is known about the function of BRCA1. Our research addresses a novel function for the BRCA1 gene product which has been largely over-looked by the rest of the scientific community. The data that we have developed during the past year holds great promise that our hypothesis was, in fact, correct (that BRCA1 is a DNA binder) and that we are very likely to generate important new data in the coming year.

In line with our Statement of Work, we plan to do the following:

1. Examine identified immunopurified clones for binding by BRCA1 protein. This will be accomplished by gel shift and DNase I footprinting assays.
2. Determine whether a mutant BRCA1 protein (such as one containing a missense mutation in the RING finger domain) is able to bind isolated DNA fragments and activate reporter gene expression.
3. Screen additional pools in our library of immunopurified fragments for BRCA1-dependent promoter activity.

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APPENDICES

Table 1. Luciferase reporter assays for pools 1-30.

Pool #	Experiment #1 BRCA1 over-expression -/+ (fold induction)	Experiment #2 BRCA1 over-expression -/+ (fold induction)
1	70/109 (1.5)	
2	59/103 (1.7)	
3	34/89 (2.6)	19/95 (5.0)
4	37/79 (2.1)	40/85 (2.1)
5	44/57 (1.3)	
6	32/29 (0.9)	
7	40/63 (1.5)	
8	21/33 (1.5)	
9	19/22 (1.1)	
10	12/12 (1.0)	
11	57/68 (1.2)	
12	19/30 (1.6)	
13	64/62 (0.96)	
14	49/62 (1.2)	
15	54/71 (1.3)	
16	26/31 (1.2)	
17	40/53 (1.3)	
18	20/16 (0.8)	
19	37/56 (1.5)	
20	20/22 (1.1)	
21	99/107 (1.1)	
22	81/92 (1.1)	
23	37/74 (2.0)	8.2/59 (7.1)
24	30/54 (1.8)	
25	60/201 (3.3)	30/122 (4.0)
26	38/99 (2.6)	32/77 (2.4)
27	9/15 (1.6)	
28	22/35 (1.6)	
29	90/150 (1.65)*	36/71 (2.0)
30	53/65 (1.2)	

Legend to Table 1. Pool DNA containing cloned immunopurified DNA fragments was co-transfected either with BRCA1 or an empty expression plasmid. Each number represents the mean of two separate transfections. Pools that gave a level of luciferase induction of 2.0 or greater were analyzed a second time (Experiment 2). *Pool number 29 was analyzed a second time owing to the high level of luciferase expression observed in experiment #1.

Table 2. Luciferase reporter assays for individual clones from pools 3 and 4.

Clone #	Pool 3 BRCA1 over-expression -/+ (fold induction)	Pool 4 BRCA1 over-expression -/+ (fold induction)
1	3.9/6.1 (1.5)	4/5 (1.2)
2	0.4/1.6 (3.8)	27/38 (1.4)
3	56/60 (1.1)	53/52 (1.0)
4	22/26 (1.2)	80/66 (0.8)
5	1/1.8 (1.7)	112/135 (1.2)
6	2.5/5.4 (2.1)	22/20 (1.0)
7	28/26 (0.9)	960/1445 (1.5)
8	11/25 (2.1)	49/40 (0.8)
9	308/1010 (3.2)	8/15 (1.8)
10	4.9/13.6 (2.7)	31/102 (3.3)
11	25/52 (2.0)	290/394 (1.3)
12	13/20 (1.5)	51/60 (1.1)
13	23/58 (2.5)	48/97 (2.0)
14	35/41 (1.2)	10/17 (1.7)
15	37/45 (1.2)	173/258 (1.5)
16	10/14 (1.5)	13/20 (1.5)

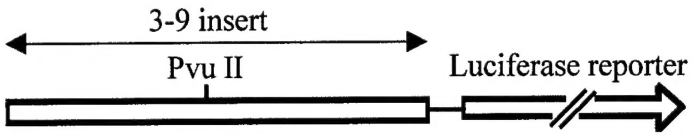
Legend to Table 2. Individual plasmid clones from pools 3 and 4 were co-transfected either with BRCA1 or an empty expression plasmid. Each number represents the mean of two separate transfections.

Table 3. Summary of transcription factor binding sites present in sequenced clones.

	AP2	AP4	c-Ets-1(p54)	DELTA EF1	GC	GKLF	Ik2	Sp1
3-9	21	7	19	9	25	9	23	29
4-10	1	9	5	5	0	7	13	0
26-13*	3	13	14	5	4	18	35	4

Legend to Table 3. Transcription factor binding sites were identified by the MatInspector program on the Internet. Only highest scoring sites (with greater than 5 hits in the 3-9 sequence) are shown. Abbreviations: AP2, activator protein 2; AP4, activator protein 4; c-Ets-1, murine Ets-1 (p54); DELTA EF1, deltaEF1; GC, GC box elements; GKLF, gut-enriched Krueppel-like factor; Ik2, Ikaros 2; Sp1, stimulating protein 1. *Clone 26-13 was identified by hybridization using the insert from 3-9 as the probe; the summary of binding sites for this clone is derived from partial sequence information (see text).

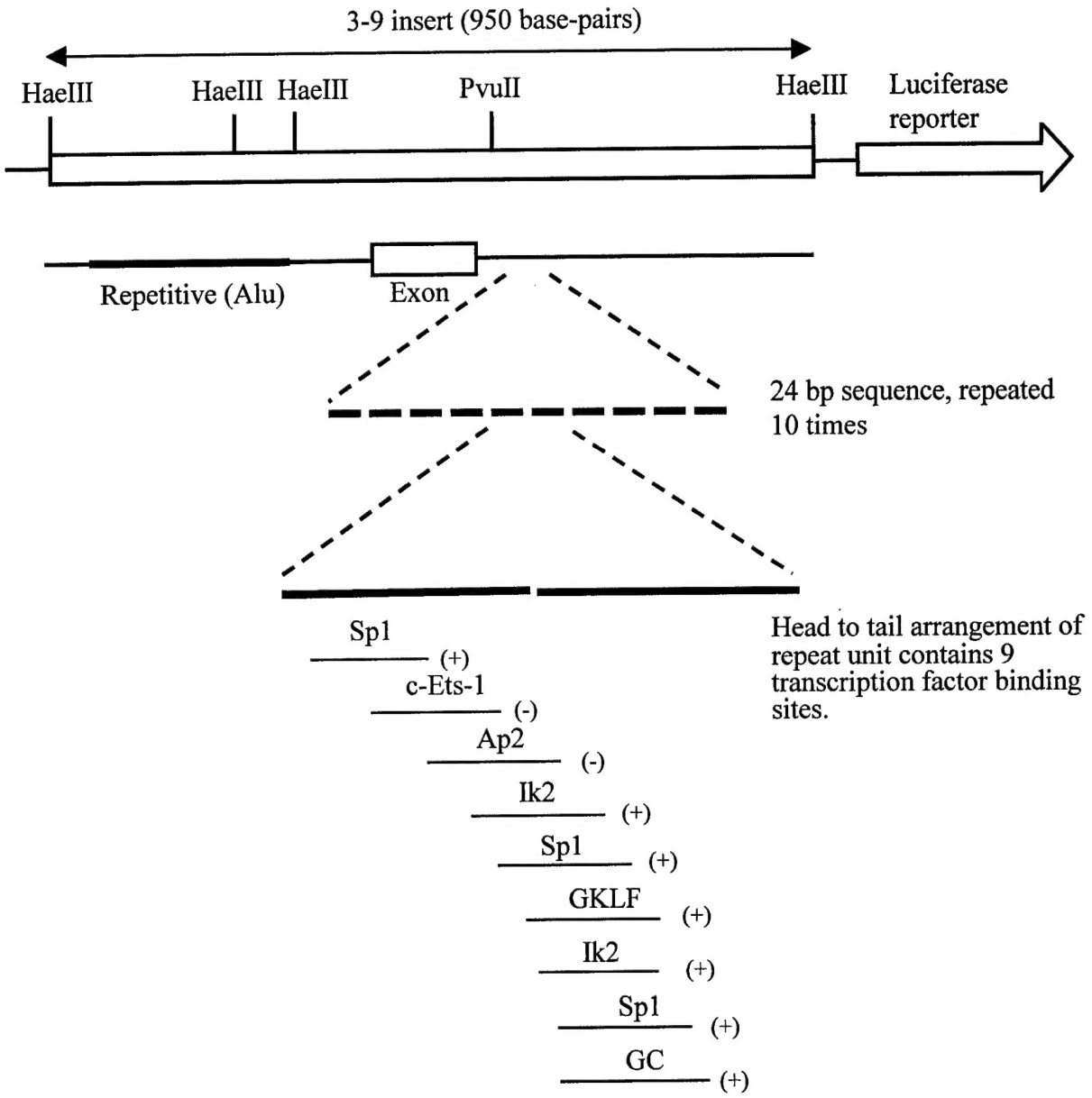
Figure 1. Refining the promoter region of clone 3-9.



from the 3-9 insert:		
BRCA1 over-expression -/+ (fold):	89/123 (1.4)	206/422 (2.0)

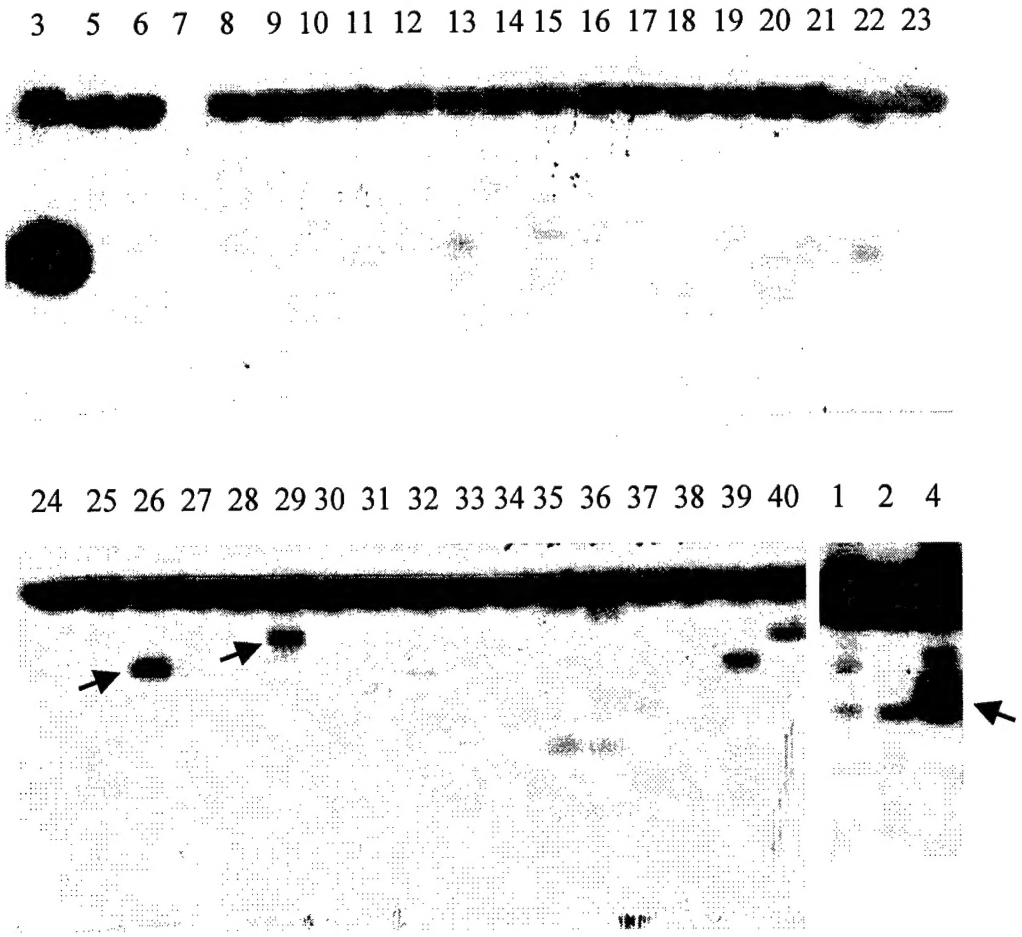
Legend to Figure 1. The insert of clone 3-9 was digested with Pvu II and deletion constructs, removing DNA from either side of the Pvu II site, generated. The resulting plasmids were transfected with or without BRCA1 cDNA and assayed for luciferase activity. Numbers represent the mean of two experiments.

Figure 2. Sequence analysis of clone 3-9.



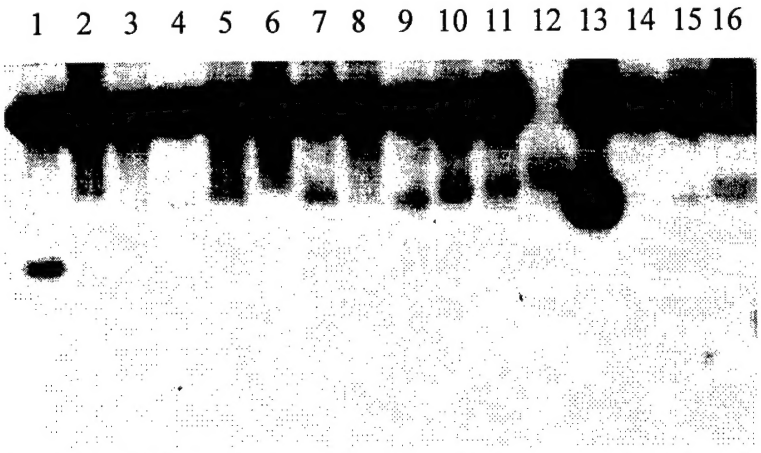
Legend to Figure 2. Sequence analysis of the 3-9 clone revealed that it contained 10 repeats each of 24 bp. The repeats are located in the portion of the 3-9 insert responsible for BRCA1-regulated promoter activity. In total, the repeat structure contains 74 transcription factor binding sites; (+) and (-) indicate the direction of the DNA strand containing the appropriate binding site.

Figure 3. Hybridization of DNA from pools 1-40 with the 450 bp promoter fragment from clone 3-9.



Legend to Figure 3. Plasmid DNA from the first 40 pools of our library was digested with Kpn I and Hind III, to release insert DNAs, and blotted onto nitrocellulose. The membrane was hybridized with the 450 bp Pvu II-Hind III fragment from clone 3-9. Note that the strongly hybridizing band present in each lane is vector DNA since the probe contained a small piece of multiple cloning site.

Figure 4. Hybridization of individual clones from pool 26 with the promoter fragment from clone 3-9.



Legend to Figure 4. Individual plasmid clones from pool 26 were analyzed as described in the legend to Figure 3.